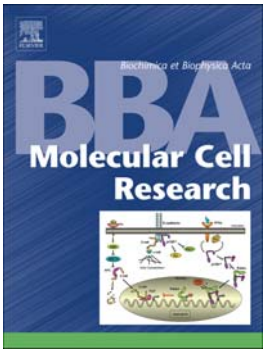

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PII: S0167-4889(16)30319-6
DOI: doi: 10.1016/j.bbamcr.2016.11.024
Reference: BBAMCR 17995

To appear in: *BBA - Molecular Cell Research*

Received date: 23 September 2016
Revised date: 16 November 2016
Accepted date: 23 November 2016

Please cite this article as: Tamara Vervloessem, Hristina Ivanova, Tomas Luyten, Jan B. Parys, Geert Bultynck, The selective Bcl-2 inhibitor venetoclax, a BH3 mimetic, does not dysregulate intracellular Ca^{2+} signaling, *BBA - Molecular Cell Research* (2016), doi: 10.1016/j.bbamcr.2016.11.024

The selective Bcl-2 inhibitor venetoclax, a BH3 mimetic, does not dysregulate intracellular Ca^{2+} signaling

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Abstract

Anti-apoptotic B cell-lymphoma-2 (Bcl-2) proteins are emerging as therapeutic targets in a variety of cancers for precision medicines, like the BH3-mimetic drug venetoclax (ABT-199), which antagonizes the hydrophobic cleft of Bcl-2. However, the impact of venetoclax on intracellular Ca^{2+} homeostasis and dynamics in cell systems has not been characterized in detail. Here, we show that venetoclax did not affect Ca^{2+}-transport systems from the endoplasmic reticulum (ER) in permeabilized cell systems. Venetoclax (1 μM) did neither trigger Ca^{2+} release by itself nor affect agonist-induced Ca^{2+} release in a variety of intact cell models. Among the different cell types, we also studied two Bcl-2-dependent cancer cell models with a varying sensitivity towards venetoclax, namely SU-DHL-4 and OCI-LY-1, both diffuse large B-cell lymphoma cell lines. Acute application of venetoclax did also not dysregulate Ca^{2+} signaling in these Bcl-2-dependent cancer cells. Moreover, venetoclax-induced cell death was independent of intracellular Ca^{2+} overload, since Ca^{2+} buffering using BAPTA-AM did not suppress venetoclax-induced cell death. This study therefore shows that venetoclax does not dysregulate the intracellular Ca^{2+} homeostasis in a variety of cell types, which may underlie its limited toxicity in human patients. Furthermore, venetoclax-induced cell death in Bcl-2-dependent cancer cells is not mediated by intracellular Ca^{2+} overload.

Key words

venetoclax; ABT-199; Bcl-2; BH3 mimetic; calcium; IP_3 receptor

Highlights

- Venetoclax does not affect ER Ca^{2+}-transport systems.
- Venetoclax fails to trigger Ca^{2+} release by itself in a variety of cell models.
- Venetoclax does not affect the agonist-induced Ca^{2+} release in a variety of cell models.
- Venetoclax-induced cell death in Bcl-2-dependent cancer is Ca^{2+} independent.
- Venetoclax does not adversely affect intracellular Ca^{2+} homeostasis.

Abbreviations

7-AAD:	7-aminoactinomycin D
Bcl-2:	B cell lymphoma-2
BCR:	B-cell receptor
BH:	Bcl-2 Homology
BIRD-2:	Bcl-2/IP_3 receptor Disrupter-2
CLL	Chronic lymphocytic leukemia
DLBCL	Diffuse large B-cell lymphoma
ER:	Endoplasmic reticulum
IICR	IP_3-induced calcium release
IP_3:	inositol 1,4,5-trisphosphate
IP_3R:	inositol 1,4,5-trisphosphate receptors
SERCA:	sarco/endoplasmic reticulum Ca^{2+} ATPase

1. Introduction

A major regulator of apoptotic cell death is the B cell lymphoma-2 (Bcl-2) family of proteins, consisting of pro- and anti-apoptotic members. They all share at least one of the four Bcl-2 Homology (BH1-4) domains. The anti-apoptotic members like Bcl-2 and Bcl-Xl possess all four BH domains, while the pro-apoptotic members either contain only the BH3 domain, like Bim and Bad, or consist of the BH1-3 domains, like Bax and Bak. Bcl-2 proteins control apoptosis by a complex BH3-dependent interaction network. The BH1-3 domains of the anti-apoptotic proteins form a hydrophobic cleft, which neutralizes the BH3 domain of the pro-apoptotic members, preventing Bax/Bak activation and cell death [1-4].

A hallmark of cancer cells is their ability to avoid apoptosis, despite on-going oncogenic stress. In many cancers this effect is a result of dysregulation within the Bcl-2 family, such as overexpression of the anti-apoptotic members, including Bcl-2 [5, 6]. Thus, these cells, designated as Bcl-2-dependent cancer cells, are addicted to Bcl-2 for their survival despite high levels of pro-apoptotic proteins like the BH3-only protein Bim. Therefore, Bcl-2 antagonism has been shown to be a promising therapeutic avenue for these cancers [7, 8]. An important class of Bcl-2 inhibitors are the BH3-mimetic drugs [9-11], including ABT-737 [12], its orally bioavailable derivative ABT-263 (navitoclax) [13] and ABT-199 (venetoclax) [14]. These small molecules are anti-cancer agents that target the hydrophobic cleft of anti-apoptotic Bcl-2 proteins, liberating the BH3-only proteins and thus activating Bax/Bak-dependent apoptosis. ABT-737, which inhibits Bcl-2, Bcl-Xl and Bcl-w, was shown to trigger apoptosis in various lymphoid malignancies [15-17] and in solid tumors [12, 18]. However, a caveat for the clinical use of ABT-737 and its orally bioavailable analogue ABT-263 were their adverse impact on platelet survival [18]. To overcome these limitations and adverse effects, a highly selective Bcl-2 inhibitor venetoclax, which spares platelets, has been developed [14]. Venetoclax induces apoptosis in Bcl-2-dependent cancer cells by selectively antagonizing Bcl-2 with high affinity (K_i < 0.01 nM; [14]).

Besides scaffolding pro-apoptotic Bcl-2-family members, anti-apoptotic Bcl-2 proteins also act through Ca^{2+}-signaling modulation, a feature common among several oncomodulators [19, 20]. Bcl-2 directly targets different Ca^{2+}-flux systems, including intracellular Ca^{2+}-release channels [21, 22]. As such, Bcl-2 can directly bind to inositol 1,4,5-trisphosphate receptors (IP_3Rs), thereby suppressing pro-apoptotic Ca^{2+} fluxes from the ER [21, 23]. IP_3R/Bcl-2-complex formation underlies the survival of a variety cancer cell types, including B-cell cancer cells [24, 25], lung cancer cells [26] and multiple myeloma [27]. At the molecular level, a primary role for Bcl-2's inhibitory impact on IP_3R channels has been attributed to its

BH4 domain and its C-terminal transmembrane domain [28-30]. The latter mediates the efficient *in cellulo* inhibition of IP_3Rs. The inhibition of IP_3Rs by Bcl-2 seems to be independent of Bcl-2's hydrophobic cleft, since antagonizing this functional domain did not interfere with Bcl-2's ability to inhibit IP_3R function [30].

Importantly some Bcl-2 antagonists have been demonstrated to affect intracellular Ca^{2+} handling [31-33]. Nevertheless, the acute impact of venetoclax on intracellular Ca^{2+}-signaling events originating from the ER has not yet been characterized in detail. Here, we studied the effect of venetoclax on Ca^{2+} signaling in permeabilized and intact cell systems at micromolar concentrations, which are well above the K_i for Bcl-2 inhibition (<0.01 nM) and LD_{50} values for several Bcl-2-dependent cancer types, including primary chronic lymphocytic leukemia (CLL) cells (10 nM range) [14, 34]. Venetoclax did not impact the ER Ca^{2+}-uptake and -release mechanisms in permeabilized cells. In addition, acute application of venetoclax did neither trigger cytosolic Ca^{2+} release by itself nor affect agonist-induced Ca^{2+}-release in a variety of intact cell models, including two Bcl-2-dependent diffuse large B-cell lymphoma (DLBCL) cell lines. In Bcl-2-dependent cancer cells, venetoclax-induced cell death was not suppressed by intracellular Ca^{2+} buffering, further indicating that venetoclax does not act through triggering intracellular Ca^{2+} overload.

2. Materials and Methods

2.1. Cells

SU-DHL-4 and OCI-LY-1 DLBCL cell lines were kindly obtained from Dr. Anthony Letai (Dana-Farber Cancer Institute, Boston, Massachusetts, USA), who performed a complete "BH3 profiling" analysis of these cell lines. These B-cell lines were authenticated by the University of Arizone Genetics Core (UAGC, Tucson, AZ) *via* Science Exchange (www.scienceexchange.com) using autosomal short tandem repeat (STR) profiling. The results were validated using reference databases such as DSMZ (Germany) and sample profiles (allelic values) and electropherogram trace data were provided. All cell lines displayed a perfectly matched profile with 8 tested alleles (8/8). The SU-DHL-4 cell line was cultured in suspension in RPMI-1640 media. The OCI-LY-1 DLBCL cell line was cultured in suspension in Iscove modified Dulbecco medium (Invitrogen, Merelbeke, Belgium). Both DLBCL cell lines were cultured at 37 °C and 5% CO_2. MEF cells were cultured at 37°C in a 10% CO_2 incubator in DMEM/Ham's F12 medium supplemented with 10% fetal calf serum. Wehi 7.2 and Wehi 7.2 overexpressing Bcl-2 were cultured in 10% DMEM + G418 (1000 µg/ml) in 37°C in a 10% CO_2. COS-1 cells were cultured at 37°C, 10% CO_2 in DMEM,

containing 2.5 μg/ml fungizone. HeLa cells were grown in Glutamax-containing DMEM supplemented with 10 mM HEPES buffer. The cells were grown at 37°C and 5% CO_2. HEK293 cells were grown in DMEM containing 4500 mg/L glucose and 50 μg/mL gentamicin. These cells were cultured at 37°C and 5% CO_2. All media were supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (100 × GlutaMAX, Gibco/Invitrogen, Merelbeke, Belgium) and penicillin and streptomycin (100 × Pen/Strep, Gibco/Invitrogen, Merelbeke, Belgium).

2.2. Reagents

Venetoclax (purity > 99.5%) was purchased from ChemieTek (Indianapolis, USA) and AffiniPure F(ab')2 Fragment Goat Anti-Human IgG + IgM (H+L) (IgG/IgM) was obtained via SANBIO (Ca, USA). Hamster anti–mouse CD3 ε chain monoclonal antibody (clone 145-2C11) was obtained from BD Bioscience. Venetoclax stock solutions were prepared at a final concentration of 10 mM in 100% DMSO. DMSO was used in control experiments (vehicle-treated condition) at a concentration correlating with the DMSO concentration present in the condition with the highest venetoclax concentration.

2.3. Unidirectional $^{45}Ca^{2+}$-flux assay

The unidirectional $^{45}Ca^{2+}$-flux experiments were performed in permeabilized HeLa and MEFs as previously described [35]. Venetoclax (ABT-199) or the vehicle (DMSO) were added during the loading with $^{45}Ca^{2+}$ to monitor the effect on the uptake. The $^{45}Ca^{2+}$-efflux experiments were performed in presence of thapsigargin (4 μM) to prevent sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) activity. IP_3-induced Ca^{2+}-release (IICR) was triggered during the unidirectional $^{45}Ca^{2+}$-efflux phase by the addition of 0.7 μM or 3 μM IP_3 for 2 min in Hela or MEFs respectively. Venetoclax or the vehicle (DMSO) were added 2 (in Hela) or 4 (in MEF) min before IP_3 till 2 min after IP_3. IICR was plotted as fractional loss, representing the amount of Ca^{2+} leaving the store in a 2-min time period divided by the total store Ca^{2+} content at that time point as a function of time.

2.4. Cytosolic Ca^{2+} measurements in intact cells

Intact cells were loaded for 30 min with 1.25 μM Fura-2 AM at room temperature in modified Krebs solution (150 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 11.6 mM HEPES (pH 7.3), 11.5 mM glucose and 1.5 mM $CaCl_2$). Afterwards, the cells were incubated for at least 30 minutes in the absence of Fura-2 AM, as $4x10^5$ lymphocytes (DLBCL, WEHI 7.2 and WEHI 7.2 Bcl-2

cells) were seeded in poly-L-lysine coated 96-well plates (Greiner). Adherent cells; COS-1 (10 000 cells), HeLa (10 000 cells), HEK-293T (150 000 cells), MEF (2 000 cells) were seeded in a 96-well plate. Fluorescence was monitored on a FlexStation 3 microplate reader (Molecular Devices) by alternately exciting the Ca^{2+} indicator at 340 and 380 nm and collecting emission fluorescence at 510 nm.

2.5. Mitochondrial Ca^{2+} measurements in intact cells

24 h after seeding (15 000 cells in a chamber), HeLa cells were transfected with pCMV24-CEPIA3mt vector (Addgene plasmid #58219), originally developed by Iino and co-workers [36]. The measurements were performed 48 h after transfection in modified Krebs solution, using Zeiss Axio Observer Z1 Inverted Microscope equipped with a × 20 air objective (filter set 62 HE, excitation at 474 ± 28 nm and emission 527 nm) and a high-speed digital camera (Axiocam Hsm, Zeiss, Jena, Germany).

2.6. Apoptosis assay

OCI-LY-1 and SU-DHL-4 cells (5×10^5 cells/ml) were treated for 4 h with venetoclax. Thereafter, the cells were incubated with Annexin V-FITC (Life Technologies, Brussels, Belgium) and 7-aminoactinomycin D (7-AAD) (Life Technologies, Brussels, Belgium) for 15 min. Cell death was analyzed by quantifying the population of Annexin V-FITC-positive and 7-AAD positive cells with an Attune® Acoustic Focusing Flow Cytometer (Applied Biosystems, Brussels, Belgium).

3. Results

3.1. Venetoclax does not affect ER Ca^{2+}-uptake and -release mechanisms in permeabilized cells.

To assess the ability of venetoclax (ABT-199) to modulate the ER Ca^{2+}-uptake activity in the absence of plasmalemmal and mitochondrial Ca^{2+} fluxes and of IICR, we used the highly quantitative $^{45}Ca^{2+}$-flux assay on permeabilized cells. The ER stores of saponin-permeabilized HeLa cells were loaded with $^{45}Ca^{2+}$ while different concentrations of venetoclax (up to 30 μM) were added during the loading phase. In these experiments, venetoclax did not influence the steady-state $^{45}Ca^{2+}$ loading levels (**fig. 1A**). The application of venetoclax during the passive efflux phase also did not affect the ER Ca^{2+}-leak rate (**fig. 1B**).

Next, we also monitored the direct effect of venetoclax on the Ca^{2+}-flux properties of the IP_3R by measuring the unidirectional $^{45}Ca^{2+}$ flux on permeabilized cells in the presence of IP_3. After loading the non-mitochondrial stores of HeLa cells with $^{45}Ca^{2+}$, Ca^{2+} release was

induced by application of 0.7 μM IP_3. **Fig. 1C&D** shows that venetoclax does not have a significant effect on IICR. We also scrutinized the impact of venetoclax in mouse embryonic fibroblasts (MEFs), which have very low endogenous levels of Bcl-2 in order to assess any Bcl-2-independent effects of venetoclax on IICR. Also in this cell system venetoclax, applied at 1 μM, did not affect IICR triggered by various [IP_3] (**fig. 1E**).

3.2. In intact cells, venetoclax does not dysregulate intracellular Ca^{2+} signaling in a variety of cell types, including Bcl-2-dependent cancer cell lines.

We assessed the effect of acute venetoclax treatment on cytosolic Ca^{2+} signals in different cell line models. We screened six different cell models of different origins (human, monkey, mouse) and types (fibroblasts, cervical epithelial cells, immature T cells, kidney epithelial cells). Several adherent cell lines (**fig. 2**) and suspension cell lines (**fig. 3**) were loaded with the ratiometric Ca^{2+} dye Fura-2 AM and 1 μM venetoclax was applied 30 seconds after starting the measurement. After 10 minutes, an agonist (5 μM ATP or 20 μg/ml anti-CD3 respectively) was used to trigger IICR. Venetoclax did neither induce cytosolic [Ca^{2+}] rises by itself nor affected agonist-induced [Ca^{2+}] rises in these different cell models as measured by the peak amplitude in MEF (**fig. 2A**), COS-1 (**fig. 2B**), HEK293-T (**fig. 2C**) and HeLa cells (**fig. 2D**). We also used WEHI7.2 cells, since these cells express very low endogenous levels of Bcl-2 [23], allowing to assess Bcl-2-independent impacts of venetoclax on Ca^{2+} homeostasis. Also, in WEHI7.2 cells, venetoclax did neither impact basal Ca^{2+} levels nor impact anti-CD3-induced Ca^{2+} release (**fig. 3A**). Moreover, we also determined the ability of venetoclax to alleviate the inhibition of IP_3Rs by Bcl-2. We therefore use WEHI7.2 cells overexpressing Bcl-2. Consistent with previous reports [23, 37], WEHI7.2 cells overexpressing Bcl-2 displayed a dampened Ca^{2+} response to anti-CD3 compared to native WEHI7.2 cells (**fig. 3A and fig. 3B**). Moreover, addition of venetoclax did not alleviate the inhibition of IP_3R-mediated Ca^{2+} flux by Bcl-2 (**fig. 3B**), which is consistent with our previous data obtained in COS cells transiently overexpressing Bcl-2 [30].

In addition to this, we tested whether acute application of venetoclax is able to affect the mitochondrial Ca^{2+} flux, since prolonged incubation of ovarian cancer cells with ABT-737, another BH3-mimetic drug, leads to alterations in ER-mitochondrial Ca^{2+} signaling [38]. Using the genetically encoded Ca^{2+}indicator CEPIA specifically targeted to the mitochondria [36], we monitored the mitochondrial Ca^{2+} signals in intact HeLa cells exposed to 1 μM venetoclax or vehicle treatment **(fig. 4)**. Similarly to the cytosolic Ca^{2+} measurements, 10 minutes after the application of venetoclax, an agonist (25 μM ATP) was

added to trigger mitochondrial Ca^{2+} influx. At the end of the experiment, uncoupler (5 μM FCCP) was applied to induce Ca^{2+} leak from the mitochondria. Our results indicated that venetoclax did neither trigger mitochondrial Ca^{2+} mobilization by itself nor modulate the ATP-induced Ca^{2+} flux into the mitochondria (**fig. 4**).

Finally, two different Bcl-2-dependent diffuse large B-cell lymphoma (DLBCL) cell lines, namely SU-DHL-4 and OCI-LY-1 cells, were assessed. These cancer cells are addicted to Bcl-2 for their survival and accordingly are primed for cell death [39]. These cells can be killed by venetoclax, although with different sensitivity (SU-DHL-4 EC_{50} = 7.189 μM and OCI-LY-1 EC_{50} = 0.026 μM, [14]). Yet, acute addition of 1 μM venetoclax did not lead to an increase in the fluorescence of Fura-2 in either of the cell lines, while both cell lines showed an increase in cytosolic Ca^{2+} when the B-cell receptor agonist, IgG/IgM, was added **(fig. 5A-B top)**. Moreover, we calculated the Ca^{2+} peak amplitude after the addition of IgG/IgM for each condition, which did not significantly differ from the vehicle control in the two cell lines, meaning that venetoclax by itself did not modulate B-cell receptor (BCR)-induced Ca^{2+} signals in these DLBCL cell lines **(fig. 5A-B bottom)**. Furthermore, we also applied two different submaximal concentrations of IgG/IgM to SU-DHL-4 cells, but again the Ca^{2+} peak amplitude was not significantly different between vehicle- and venetoclax-treated cells (**fig. 5C**). Hence, venetoclax did not sensitize the DLBCL cell line towards BCR stimulation-induced Ca^{2+} signaling.

3.3. Venetoclax-induced cell death in Bcl-2-dependent cancer cells cannot be prevented by chelating intracellular Ca^{2+}.

Finally, we determined the contribution of intracellular Ca^{2+} overload to the mechanism of venetoclax- induced cell death. Therefore, we measured apoptosis after venetoclax treatment in absence or presence of 10 μM BAPTA-AM, a cell-permeable Ca^{2+} chelator (**fig. 6**). This concentration of BAPTA-AM has been previously shown to interfere with Ca^{2+}-driven processes, e.g. suppressing BIRD-2-induced cell death [25] and preventing starvation-induced autophagy [40]. In this experimental approach **(fig. 6B)** we too validated the effectiveness of the BAPTA-AM treatment by showing that SU-DHL-4 cells treated with BAPTA-AM were less sensitive to exposure with Bcl-2/IP_3 receptor Disrupter-2 (BIRD-2), a Bcl-2 inhibitor targeting the BH4 domain of Bcl-2 and causing cell death in a Ca^{2+}-dependent manner, than SU-DHL-4 not treated with BAPTA-AM [26, 41]. Flow cytometric analysis revealed that chelating the intracellular Ca^{2+} during the complete time course did not protect SU-DHL-4 **(fig. 6B)** or OCI-LY-1 **(fig. 6A)** cells from cell death after an incubation of 4 h with

venetoclax. Please note that higher concentrations of venetoclax were used in SU-DHL-4 cells than in OCI-LY-1 cells, as the latter are much more sensitive to venetoclax treatment than the former, consistent with previous reports [14]. Of note, BAPTA-AM treatment significantly increased venetoclax-induced cell death in these cells, which may suggest that basal Ca^{2+} signaling contributes to the survival of these B-cell cancer cells, in particular when Bcl-2 is targeted with venetoclax.

4. Discussion

The major findings of this study are that (i) venetoclax (ABT-199) does not dysregulate intracellular Ca^{2+} signaling and ER Ca^{2+}-flux systems, since acute application of venetoclax does not affect the intracellular Ca^{2+} homeostasis in different cell types of a variety of origins and (ii) venetoclax-induced cell death in Bcl-2-dependent cancer cell models does not occur *via* cellular Ca^{2+} overload, since intracellular Ca^{2+} buffering did not suppress venetoclax-induced cell death in these cells.

We previously demonstrated that venetoclax did neither interfere with the inhibition of IP_3Rs by Bcl-2 nor impacted ER Ca^{2+}-store content [30]. In this study, we focused on the effects of venetoclax itself on ER Ca^{2+}-transport systems in permeabilized cells and on Ca^{2+}-signaling events in intact cells. Although higher concentrations of venetoclax than 1 µM have been applied in permeabilized cell systems, we mainly focused on the effects of 1 µM venetoclax on intracellular Ca^{2+} dynamics in intact cells. This concentration is well above the LD_{50} of venetoclax in a variety of cancer cells, including CLL (LD_{50} of about 10 nM [34]). In permeabilized cells, venetoclax did neither impact ER Ca^{2+}-uptake properties, mediated by SERCA, nor ER Ca^{2+}-release properties, mediated either by IP_3Rs or by the so-called ER Ca^{2+}-leak channels. Consistent with our observations in permeabilized cells, acute application of venetoclax to intact cells failed to trigger cytosolic Ca^{2+} rises in a variety of cell models from human, monkey and mouse origin and cell types (fibroblasts, kidney epithelial cells, immature T lymphocytes, cervical epithelial cells and diffuse large B-cell lymphoma). We therefore can advocate that venetoclax, similarly to ABT-737 at concentrations lower than 10 µM [33], does not have off-target effects related to intracellular Ca^{2+}-transport systems. Moreover, acute application of venetoclax did also not affect the mitochondrial Ca^{2+} uptake in HeLa cells upon response to ATP. Therefore, on-target inhibition of Bcl-2 using this compound does not disturb Ca^{2+} homeostasis and dynamics, which is in striking contrast to other developed Bcl-2 inhibitors like BH3I-2' and HA14-1 [32, 33]. BH3I-2' caused a slow IP_3R/RyR-dependent Ca^{2+} release from internal stores of pancreatic acinar cells. Inhibition of

IP_3Rs and RyRs suppressed BH3I-2'-induced cell death in AR42J, an exocrine pancreatic cell line [32]. The HA14-1 compound partially depletes ER Ca^{2+} stores of human cell lines and triggers an acute Ca^{2+} rise in platelets, an effect that could be attributed to an inhibition of SERCA2b-driven ER Ca^{2+} uptake [33]. Its inhibitory impact on SERCA activity has been independently demonstrated by others using a stable analog of HA14-1, sHA14-1 [31]. In that study, SERCA2b inhibition was shown to cause ER stress and to contribute to the cell-death-inducing properties of sHA14-1 [31]. We could also exclude intracellular Ca^{2+} overload as part of the proximal pathways that contribute to the cell-death mechanisms triggered by venetoclax. Indeed, two venetoclax-sensitive DLBCL cell lines also did not display a rise in cytosolic [Ca^{2+}] upon acute addition of venetoclax. In these cells, venetoclax did also not affect the cytosolic Ca^{2+} signal elicited by physiological agonists like antibodies activating the B-cell receptor. Moreover, BAPTA-AM, an intracellular Ca^{2+} chelator, did not suppress venetoclax-induced cell death in Bcl-2-dependent cancer cells. Hence, it seems that venetoclax might be unique among stress/cell death inducers, as it does not trigger ER Ca^{2+} mobilization or impact ER Ca^{2+} homeostasis/dynamics as part of its cytotoxic mechanism. This is in contrast to several other stress inducers, including apoptotic stimuli like ceramide and chemotherapeutic/anti-cancer drugs [42, 43]. Recently, fast and constitutive changes in cytosolic Ca^{2+} levels have been proposed as early markers for the detection of cytotoxicity in response to H_2O_2, staurosporin, As_2O_3, gossypol and titanium(IV)-salane complexes in different cancer cell models [44]. Other chemotherapeutic agents, like adriamycin, have been shown to increase the Ca^{2+} levels in the ER lumen by increasing SERCA activity in a p53-dependent manner [45]. As such, chemotherapeutic drugs increase ER-mitochondrial Ca^{2+} flux in response to physiological stimuli and oxidative stress inducers, thereby increasing apoptotic sensitivity and thus the cytotoxic effect of the chemotherapeutic drug [45]. Cancer cells deficient in p53 lack this increased ER-mitochondrial Ca^{2+} flux and are more resistant to chemotherapy, a feature that can be overcome by overexpression of the mitochondrial Ca^{2+} uniporter [46]. Interestingly, venetoclax-induced cell death in CLL is independent of the presence of p53 or the p53 mutational status [47]. Thus, differences in the contribution of p53 might be another distinct feature between venetoclax and conventional chemotherapy in respect to ER-mitochondrial Ca^{2+} signaling, although this should be further examined.

Nevertheless, we cannot exclude that long-term treatment of venetoclax, particularly in Bcl-2-dependent cancers, could cause remodeling of ER-mitochondria contact sites and thus affect Ca^{2+} signaling at the ER-mitochondrial interface, thereby modulating venetoclax-induced cell death. Indeed, we found that BAPTA-AM treatment enhanced rather than suppressed

venetoclax-induced cell death in Bcl-2-dependent cancer cells, while BAPTA-AM was not toxic by itself in these cells. This may suggest a remodeling of intracellular Ca^{2+}-signaling proteins and/or Ca^{2+}-signaling compartments upon prolonged treatment with venetoclax. However, further work using longer venetoclax treatments in Bcl-2-dependent and –independent cell models is needed to clarify the underlying mechanisms and the contribution to cell death. Very interestingly, ABT-737, a non-selective Bcl-2/Bcl-XL inhibitor, can sensitize cholangiocarcinoma and ovarian cancer cells to cisplatin treatment via a mechanism that involves mitochondrial remodeling and Ca^{2+} signaling [38, 48]. Indeed, while cisplatin by itself induces mitochondrial hyperfusion in cholangiocarcinoma cells, it induces mitochondrial fragmentation and mitophagy when co-applied with ABT-737 [48]. Moreover, in cisplatin-resistant ovarian cancer cells, ABT-737 can also promote the formation of ER-mitochondrial contact sites induced by cisplatin, thereby augmenting cisplatin-induced Ca^{2+} rise in the mitochondria and apoptosis [38]. These observations further underpin the intimate link between the effective response to anti-cancer therapeutics and efficient ER-mitochondrial Ca^{2+} transfer [43, 46, 49].

In any case, a potential remodeling of ER-mitochondrial contact sites and alterations in ER-mitochondrial Ca^{2+} transfer occurring upon long-term treatments with venetoclax are more distal events in time. They likely are a consequence of Bcl-2 inhibition and may be closely related to changes in mitochondrial fusion/fission events and cell death. Here, we demonstrated that venetoclax, acutely added to Bcl-2-dependent and Bcl-2-independent cell models, does not provoke Ca^{2+} mobilization from the ER Ca^{2+} stores. It also does not cause the dysregulation of intracellular Ca^{2+} homeostasis and dynamics in response to physiological agonists that trigger Ca^{2+} release from the ER. These observations advocate against a proximal role for intracellular Ca^{2+} overload in venetoclax-induced cell death. Moreover, the lack of a direct impact and off-target effects of venetoclax on intracellular Ca^{2+}-flux systems in healthy/normal cells may underlie its limited toxicity in humans. Indeed, venetoclax has been successfully applied in several clinical trials for CLL, either as a mono-therapy or in combination with other drugs [50]. In 2016, venetoclax has been approved by the FDA for the treatment of 17p-deleted chronic lymphocytic leukemia [8, 51].

To conclude, we showed that (i) venetoclax does not have major off-target effects on ER Ca^{2+}-transport systems and thus does not adversely affect the Ca^{2+}-signaling profile for a large set of cell types and (ii) intracellular Ca^{2+} overload does not contribute to venetoclax-induced apoptosis in Bcl-2-dependent cancer cell models.

Acknowledgements

We thank Anja Florizoone and Marina Crabbe for excellent technical support.

Funding

Work performed in the authors' laboratory was supported by grants from the Research Foundation-Flanders (FWO grants 6.057.12, G.0819.13, G.0C91.14 and G.0A34.16), by the Research Council of the KU Leuven (OT grant 14/101) and by the Interuniversity Attraction Poles Program (Belgian Science Policy; IAP-P7/13). HI is a recipient of a doctoral fellowship of the FWO. TV has been supported by a research grant from Emmanuel van der Schueren of Kom op tegen Kanker.

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Figure legends

Fig. 1 Venetoclax does not affect ER-located Ca^{2+}-flux systems in permeabilized cells. A: Normalized ER $^{45}Ca^{2+}$ uptake values obtained from permeabilized HeLa cells in which steady state ER $^{45}Ca^{2+}$ loading was performed in the presence of different venetoclax concentrations. Values for vehicle-treated cells were taken as 100%. Data represent mean ± SEM of 4 independent experiments. **B:** Effect of venetoclax on the unidirectional $^{45}Ca^{2+}$ efflux in the absence of SERCA Ca^{2+}-uptake activity. Data represent the ER Ca^{2+} content (% of the vehicle control (CTR)) as a function of time (in min) and are plotted as mean ± SD of duplicate samples. A typical experiment out of 3 independent experiments is shown. **C:** Representative unidirectional $^{45}Ca^{2+}$-flux experiment demonstrating IICR in HeLa cells exposed to different concentrations of venetoclax (red bar) from 2 min before till 2 min after the addition of 0.7 μM IP_3 (black bar). The data are presented as fractional $^{45}Ca^{2+}$ loss (%/2 min) as a function of time (in min). Data are shown as mean ± SD of duplicate samples. **D:** The IICR from 4 independent experiments performed with 2 replicates per experiment, shown in **(C)** was calculated. Values for vehicle-treated cells were taken as 100%. Data represent mean ± SEM. **E:** Representative unidirectional $^{45}Ca^{2+}$-flux experiment in MEF cells. The IICR was triggered by 1, 4 or 10 μM IP_3 in presence or absence of 1 μM venetoclax. Venetoclax (red bar) was added from 4 min before till 2 min after the addition of IP_3 (black bar). The data are presented as fractional $^{45}Ca^{2+}$ loss (%/2 min) as a function of time (in min). Data represent mean ± SD of duplicate samples. Please note that x-axes of panel A and D represent a linear scale representing the log values of μM concentrations. For instance, 1 μM corresponds to log 1= 0. Thus, the 0 is indicated on the x-axis (*i.e.* log 1).

Fig. 2 Venetoclax (1 μM) does neither trigger intracellular Ca^{2+} release by itself nor affect agonist-induced Ca^{2+} release in the cytosol in adherent cell models. A-D left: Cytosolic Ca^{2+} measurements in Fura-2 AM-loaded MEF **(A)**, COS-1 **(B)**, HEK-293T **(C)** and HeLa **(D)** cells. The IICR was triggered by 5 μM ATP 10 minutes after the addition of the compound (1 μM venetoclax or vehicle control). Representative experiment of duplicate samples of 4 independent experiments is shown. **A-D right:** Analysis of the Ca^{2+} peak amplitude evoked by the addition of 5 μM ATP. Data are represented as mean ± SEM of N=4.

Fig. 3 Venetoclax (1 µM) does neither trigger intracellular Ca^{2+} release by itself nor affect agonist-induced Ca^{2+} release in native and Bcl-2-overexpressing immature T cells. A-B top: Cytosolic Ca^{2+} measurements in Fura-2 AM-loaded WEHI7.2 **(A)** and WEHI7.2 Bcl-2 **(B)** cells. The IICR was triggered by 20 µg/ml anti-CD3 10 minutes after the addition of the compound (1 µM venetoclax or vehicle control). A typical experiment conducted in duplicates of 4 independent experiments is shown. **A-B bottom:** Analysis of the Ca^{2+} peak amplitude evoked by the addition of 20 µg/ml anti-CD3. Data are represented as mean ± SEM of N=4.

Fig. 4 In HeLa cells, acute application of venetoclax (1 µM) did neither trigger mitochondrial Ca^{2+} uptake by itself nor affect agonist-induced mitochondrial Ca^{2+} uptake. A: Representative traces of mitochondrial Ca^{2+} measurements in HeLa cells transfected with mitochondrial targeted CEPIA are shown. 1 µM venetoclax or vehicle control was applied and after 10 minutes 25 µM ATP was used to trigger Ca^{2+} uptake in the mitochondria. Mitochondrial Ca^{2+} release was induced by 5 µM FCCP. **B:** Analysis of the Ca^{2+} peak amplitude evoked by the addition of 25 µM ATP. Data are represented as mean ± SEM of N=3. **C:** Analysis of the percentage of cells responding with mitochondrial Ca^{2+} uptake upon application of 25 µM ATP. Data are represented as mean ± SEM of N=3.

Fig. 5 In Bcl-2-dependent DLBCL cell lines, venetoclax (1 µM) does neither trigger intracellular Ca^{2+} release by itself nor affect agonist-induced Ca^{2+} release. A-C top: Cytosolic Ca^{2+} measurements in Fura-2 AM loaded OCI-LY-1 (**A**) and SU-DHL-4 cells **(B, C).** The BCR agonist was added 10 minutes after the addition of the compound (1 µM venetoclax or vehicle control) at the indicated concentrations. A typical experiment of duplicate samples of 3 independent experiments is represented. **A-C bottom:** Analysis of the Ca^{2+} peak amplitude evoked by the addition of IgG/IgM. Data are represented as mean ± SEM of N=3 (**C**) or N=4 (**A and B**).

Fig. 6 Venetoclax-induced cell death does not require intracellular Ca^{2+} overload as a causal/proximal event for apoptosis of Bcl-2-dependent cancer cells. A-B top: Representative histogram overlay of duplicates Annexin V-FITC-stained OCI-LY-1 (**A**) or SU-DHL-4 (**B**) cells treated for 4 h with venetoclax at the indicated concentrations of N= 5.

A-B bottom: Analysis of Annexin V-FITC/7-AAD- negative cells (living cells (%)) obtained using flow cytometric analysis of SU-DHL-4 and OCI-LY-1 cells treated with and without venetoclax and 10 µM BAPTA-AM for 4 h. For the SU-DHL-4 cells additional experiments with and without 10 µM BIRD-2 and BAPTA-AM were performed as controls. Data are represented as mean ± SEM of 5 independent experiments. Significance was obtained using a one-tailed paired t-test with * $p < 0.05$.

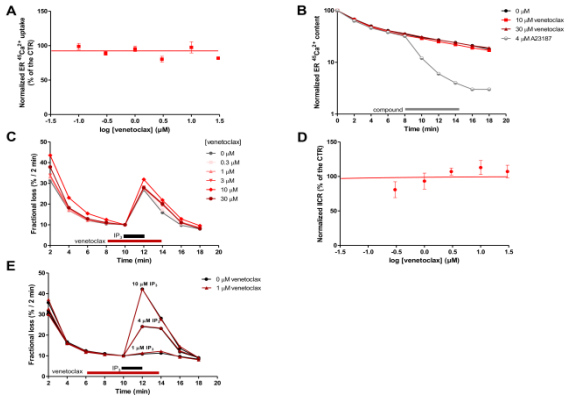

Figure 1

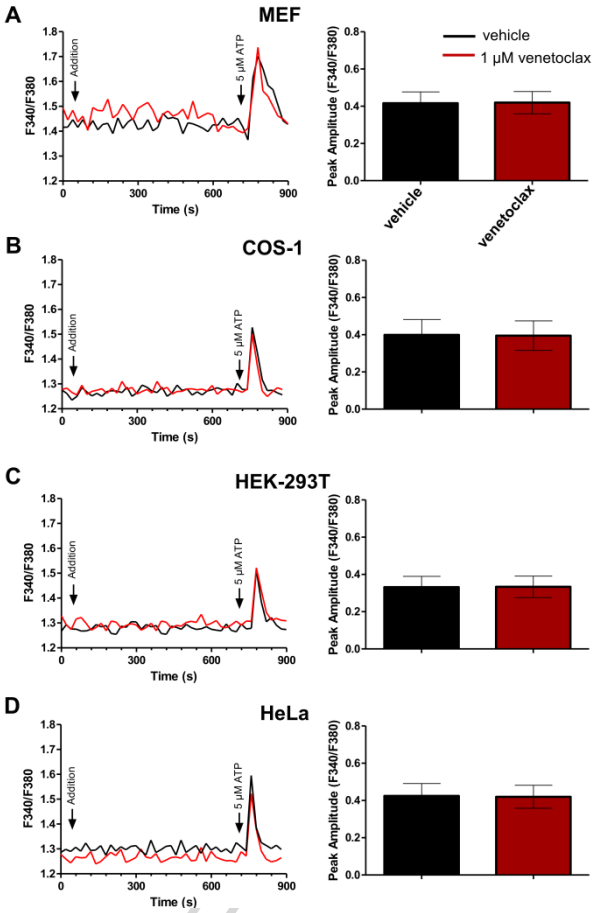

Figure 2

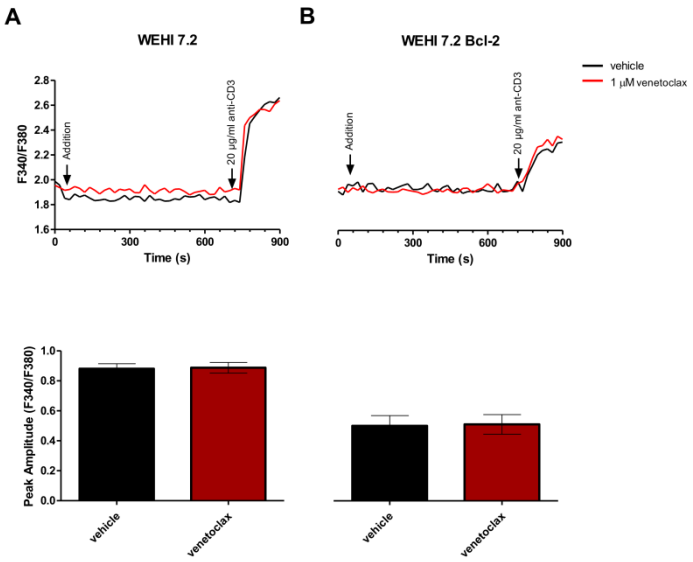

Figure 3

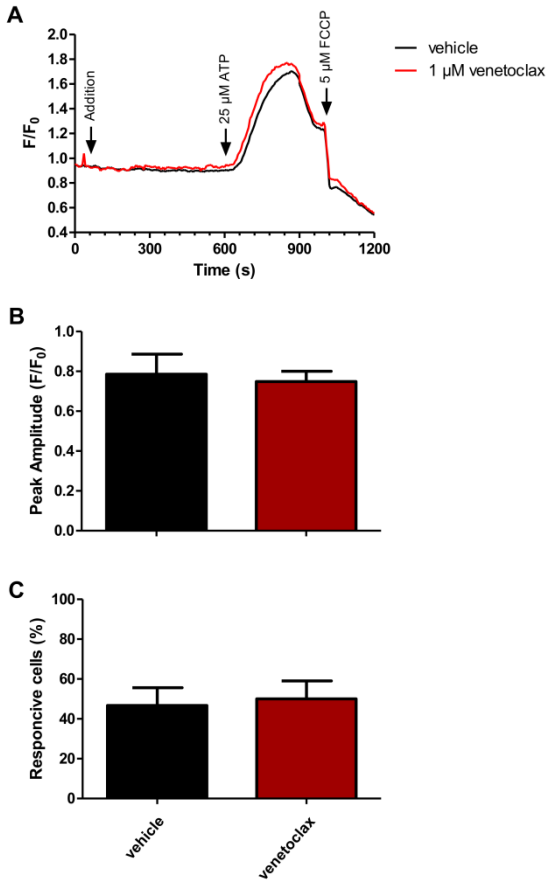

Figure 4

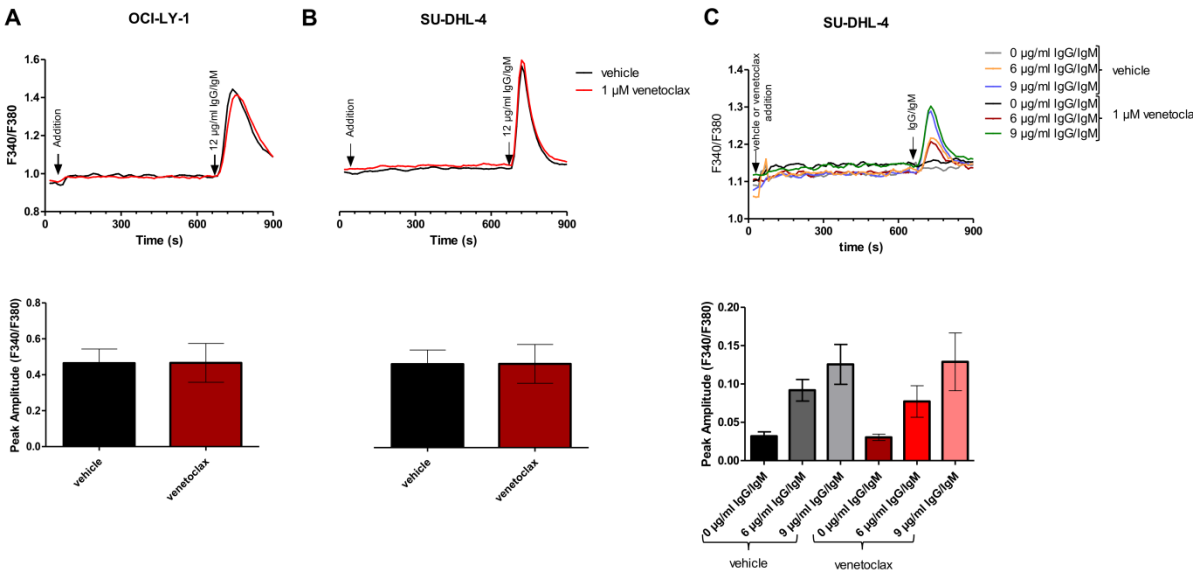

Figure 5

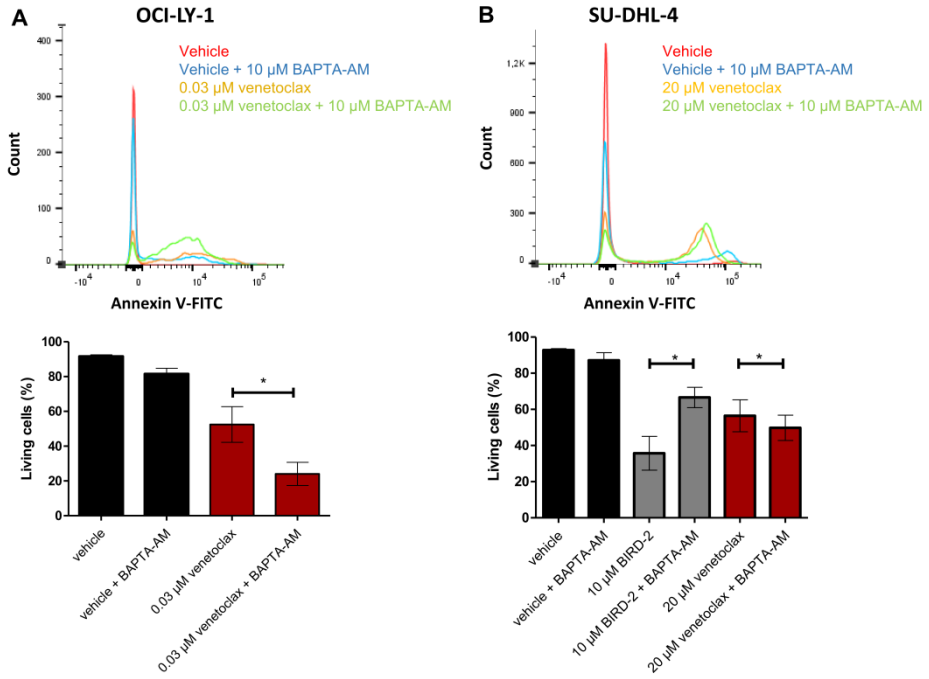

Figure 6

The authors declare that there is no conflict of interest.

Highlights

- Venetoclax does not affect ER Ca^{2+}-transport systems.
- Venetoclax fails to trigger Ca^{2+} release by itself in a variety of cell models.
- Venetoclax does not affect the agonist-induced Ca^{2+} release in a variety of cell models.
- Venetoclax-induced cell death in Bcl-2-dependent cancer is Ca^{2+} independent.
- Venetoclax does not adversely affect intracellular Ca^{2+} homeostasis